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Morphine-induced macrophage activity modulates mesangial cell proliferation and matrix synthesis

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Morphine-induced macrophage activity modulates mesangial cell proliferation and matrix synthesis. Glomerular mesangial injury is the predominant renal lesion in patients with heroin addiction. We studied the effect of morphine (an active metabolite of heroin) activated macrophages on mesangial cell (MC) proliferation and matrix synthesis. We prepared conditioned media containing either vehicle alone (CSP), macrophage secretory products (MSP) and secretory products of morphine treated macrophages (M-MSP). M-MSP increased ($P < 0.01$) the proliferation of MC when compared with MSP alone. M-MSP increased synthesis of laminin by MC at concentrations of 10 to 50% when compared with cells treated with MSP alone (at 50% concentration, MSP, 126 ± 19 vs. M-MSP, 312 ± 14 ng/mg protein, $P < 0.001$). M-MSP also increased the synthesis of collagen type IV by MC. This effect of M-MSP was attenuated ($P < 0.05$) by anti-TGF-β antibodies. Since M-MSP also increased mRNA expression for TGF-β by MC, it appears that the effect of M-MSP on MC may be mediated through the generation of TGF-β.

Focal glomerulosclerosis is the predominant glomerular lesion in patients with heroin addiction [1]. The glomerular mesangium is the main site of injury in this lesion. The mesangium consists of mesangial cells and extracellular mesangial matrix (ECM). Eighty-five to 95% of mesangial cells are similar to smooth muscle cells and the remaining 5 to 15% are bone marrow derived macrophages [2, 3]. We hypothesized that interactions between macrophages and morphine (an active metabolite of heroin) either in the blood or in the mesangium may contribute to glomerular mesangial injury.

Expansion of the glomerular mesangium has been demonstrated to be a precursor of glomerulosclerosis [2]. Mesangial expansion can occur either as a result of mesangial cell proliferation or enhanced deposition of ECM or both. Macrophages seem to play an important role in the development of mesangial expansion as shown in the experimental animal models of glomerulosclerosis [4–9]. We hypothesize that in patients with drug addiction, morphine can activate monocytes (either circulating in the blood or residing in the mesangium) to secrete specific products which may activate mesangial cells to proliferate and synthesize matrix.

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We prepared conditioned media from macrophages in the presence or absence of morphine and evaluated the effects of these conditioned media on the proliferation of mesangial cells. In addition, we determined the effects of these media on the synthesis of extracellular matrix components including laminin and collagen by mesangial cells. Recently, transforming growth factor (TGF)-β was demonstrated to play an important role in the progression of glomerulosclerosis [6]. Therefore, we also studied mRNA expression for TGF-β by mesangial cells under control as well as stimulated states. Our *in vitro* studies provide a basis for speculation in the role of morphine-macrophage interactions in heroin-induced nephropathy development.

Methods

Culture of glomerular mesangial cells

The culture of rat glomerular MC was carried out as previously described [10–12]. After three weeks in primary culture, MC were detached from the flask by adding a 0.25% trypsin EDTA solution (Sigma Chemical Co. St. Louis, MO, USA) and transferred to a plastic flask containing 5 ml of incubation medium including RPMI 1640 (Sigma), penicillin (50 U/ml), streptomycin sulfate (50 μg/ml)(Sigma) and 10% fetal calf serum (FCS). Culture flasks were kept in a 95% air - 5% CO_2 environment at 37°C. Subcultures were obtained within seven to ten day intervals by the above method. The MC that were used represent an apparently uniform cell population, as detailed in previous publications from this laboratory [10–12]. In all experiments the cells from the same primary culture were used. In these experiments MC only from the third to eighth passage were used.

Mouse mesangial cells

To have homology between mouse macrophage (Mf) secretory peptides versus mesangial cells we have carried out cell proliferation and matrix synthesis studies on mouse mesangial cells (provided by Prof. Eric G. Neilson, University of Pennsylvania, Philadelphia, PA, USA) for additional experiments. This cell line carries all the characteristics of rat mesangial cells.

Morphine (National Institute on Drug Abuse, Bethesda, MD, USA) was stocked at a concentration of 10^{-2} M in normal saline and used at a concentration of 10^{-6} M. Naloxone (NIDA) was stocked at 10^{-2} M concentration in normal saline and used in a

concentration of 10^{-6} M. Anti-TGF-β antibodies (R & D Research, Minneapolis, MN, USA) was stocked at 1 mg/ml and used in a concentration of 5 μg/ml.

Evaluation of cytotoxic effect of morphine on macrophages

To avoid the potential heterogeneity of elicited rat peritoneal macrophages, we utilized an established murine macrophage cell line J774.16 as described previously [13, 14].

To test viability of macrophages as well as cytotoxicity of the morphine treatment of macrophages we used an (-[4, 5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Thiazolyl blue) MTT reaction (Sigma). The MTT reaction is based on the ability of mitochondrial dehydrogenases to cleave the tetrazolium ring of the MTT changing the yellowish solution of the water soluble tetrazolium salt to an insoluble purple formazan. This assay measures the degree of cytotoxicity in response to a drug [15]. To determine the cytotoxic effect of morphine on macrophages, cells were plated in a 96-well plate and allowed to grow to confluence in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FCS and 1% penicillin/streptomycin. Cells were washed twice with phosphate buffered saline and then reincubated in RPMI 1640 (no phenol red) with 10% FCS ± increasing concentrations of morphine (10^{-8} M to 10^{-3} M) for 24 hours. Reconstituted MTT in an amount equal to 10% of the final concentration of media was added to each well and the plate was incubated for a further two hours. To dissolve the resulting formazan crystals MTT solubilization solution was added in an amount equal to the original volume of culture medium. The plate was then placed on a shaker for about 10 minutes to enhance the dissolution and the absorbancy was measured at 540 nm to determine differences.

Macrophage culture and collection of control and morphine-induced macrophage secretory products (conditioned media)

Macrophages were grown in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FCS and either morphine (10^{-6} M), morphine (10^{-6} M) + naloxone (10^{-6} M), or vehicle (control) on 100 mm diameter plastic culture dishes. Once macrophages reached confluence (3 to 4 days) the media was removed, cells washed three times with phosphate buffered saline (PBS) and media replaced with RPMI 1640 containing penicillin and streptomycin but without FCS. After 24 hours the supernatants were collected and spun at 1500 rpm for 10 minutes, then filtered (0.2 μm pore size acrodisc). Effects of the control and various conditioned media [containing macrophage secretory products, MSP (vehicle treated), M-MSP (morphine treated), N + M-MSP (naloxone + morphine treated)], were evaluated on MC proliferation and matrix synthesis.

Since macrophages also generate proteases which may degrade many of the secreted peptides, we prepared fresh batches of supernatants for each experiment. Some supernatants were prepared by adding protease inhibitors (100 μM 1, 10-phenanthroline and 10 μM dithiothreitol) during the incubation of macrophages in serum free RPMI (24 hr). These supernatants will be designated by using the abbreviation MϕSP (control) and M-MϕSP (morphine treated)

Fluorometric assay for morphine

A morphine fluorometric assay using the Udenfriend method [16] was performed on MSP and M-MSP to determine if M-MSP contains any residual of morphine. Briefly, equal amounts of the supernatants collected in glass conical tubes were dried under vacuum. Sulfuric acid was added to each tube which was heated at 50°C for five minutes. Five ml of water and 6 ml of ammonium hydroxide were added as the tubes continued to be heated at 50°C for two hours. The tubes were then cooled and isobutanol added. After shaking for four minutes to extract the fluorescence each sample was read at 420 emission and 365 excitation wavelengths on a spectrofluorometer.

Mesangial cell proliferation studies

Rat mesangial cells (RMC) were grown to semiconfluence on 96-well plates and then washed twice and incubated with serum-free RPMI 1640 containing 0.5% BSA and 1% insulin, transferrin and selenium solution for 72 hours to arrest growth. To determine the effects of various types of conditioned and control media on MC proliferation, growth arrested RMC were washed twice with PBS and incubated with RPMI (without FCS) either alone (CSP) or containing 50% of various conditioned media for 48 hours. In previously reported experiments we observed that 50% macrophage conditioned media provided optimal growth of mesangial cells [13, 14]. An aliquot of RPMI containing 0.25 μCi of [^{3}H]thymidine was added to each well. Six sets of experiments were performed in triplicate.

To grade the mitogenicity of various conditioned media in comparison to FCS, subconfluent RMC were growth arrested for 72 hours and washed thrice with PBS. Subsequently, cells were incubated either with RPMI 1640 alone (CSP), RPMI 1640 containing 1% FCS (FCS), MSP containing 1% FCS (FCS-MSP), or M-MSP containing 1% FCS (FCS-MMSP) for 48 hours. An aliquot of RPMI containing 0.25 μCi of [^{3}H]thymidine was added to each well.

All incubations were carried out at 37°C. At the end of the incubation period cells were washed three times with cold PBS, 200 μl of 5% trichloroacetic acid (TCA) added to each well and cells incubated at 4°C for 60 minutes. The TCA supernatant was removed and 200 μl of 0.25 N NaOH was added to each well. The cells were kept at room temperature overnight after which the contents of each well were counted in a scintillation counter. Results of thymidine incorporation were used as a measure of cellular proliferation.

To determine the effect of morphine-MϕSP on mouse mesangial cell growth, equal numbers of mesangial cells were plated on 24-well plates and allowed to grow to subconfluence. Cells were growth arrested for 72 hours. Subsequently cells were washed twice with PBS and incubated in RPMI containing variable concentrations (5%, 10% and 20%) of either MSP or M-MSP for 48 hours. At the end of the incubation period cells were washed, trypsinized and counted in a hemocytometer.

To determine the role of TGF-β in the M-MSP-induced MMC proliferation we plated MMC in 24-well plates. After three to four days, subconfluent MC were growth arrested for 72 hours. Subsequently cells were washed twice with PBS and incubated with RPMI containing either buffer alone (control), 20% MSP, 20% M-MSP, 20% M-MSP + anti-TGF-β antibody (5 μg/ml), or anti-TGF-β antibody (5 μg/ml) for 48 hours. At the end of the incubation period, cells were washed, trypsinized and counted in a hemocytometer. Three sets of experiments (each in triplicate) were carried out.

To determine the role of naloxone, an opiate receptor ligand, in

M-MSP induced MC proliferation, subconfluent MMC were growth arrested for 72 hours. Cells were washed twice with PBS and incubated in RPMI containing either 20% MSP, 20% M-MSP, or 20% N-M-MSP for 48 hours. At the end of the incubation period, cells were trypsinized and counted in a hemocytometer. Three series of experiments were performed, each in triplicate.

Laminin synthesis

The dot blot immunoassay to determine production of laminin by RMC treated with control and morphine-treated macrophage conditioned media was performed using the method of Ayo et al [17] and Madri, Pratt and Tucker [18]. In brief, MC grown for two weeks under variable experimental and control conditions were solubilized, vortexed, centrifuged and a 200 μl aliquot placed on a 0.45 μm nitrocellulose filter on the dot blot apparatus. In addition, 1 ng to 100 ng standards of laminin were applied in triplicate. The membrane was incubated for 30 minutes in Tris-buffered saline + 0.1% Tween 20 (TTBS). A dilution of rabbit anti-laminin antibody (Sigma) was added to the solution (1:1000) and incubation was continued for one hour. After washing for 15 minutes in TTBS, a secondary biotinylated antibody was added at a concentration of 5 μg/ml for a one hour incubation. Following a second 15-minute wash, Vectastain Elite, an immunoperoxidase system, was used prior to staining with 4-chloro-naphthol. Relative absorbancies were measured on an LKB 2202 ultrascan densitometer attached to an LKB 2220 integrator. A standard curve was established and laminin concentration determined.

To determine the effect of macrophage supernatant and morphine treated macrophage supernatant on laminin synthesis, equal numbers of RMCs were plated in six-well culture plates in incubation medium containing RPMI 1640, 2.5% FCS and 50% supernatants (MSP and M-MSP) for two weeks. Media was changed every third day. At the end of the incubation, cells were washed three times with PBS and scraped for the laminin assay. An aliquot of scraped cells was used to measure protein by the method of Bradford [19]. Four sets of experiments were carried out, each in quadruplicate. Production of laminin was calculated as ng/μg or mg of cellular protein.

To evaluate the comparable dose response of MSP and M-MSP, equal numbers of RMCs were plated on six-well plates in incubation medium containing RPMI 1640, 2.5% FCS and variable concentrations (10 to 70%) of MSP or M-MSP for two weeks. Media was changed twice a week. At the end of two weeks, cells were washed thrice with PBS and scraped from each well. Measurement of the total amount of laminin and cell protein was carried out as described above.

To prevent the degradation of peptides in macrophage supernatants we collected supernatants (MϕSP or M-MϕSP) in the presence of protease inhibitors. In order to provide homology to the mouse macrophage peptides, we used mouse mesangial cells in these additional experiments. In these experiments, equal numbers of mouse mesangial cells were plated in six-well plates. Incubation media contained RPMI + 5% FCS + 10% various control and macrophage supernatants (prepared in the presence of protease inhibitor). Cells were incubated in media containing either control or macrophage supernatants for one week. Incubation medium was changed every other day. At the end of the incubation period cells were washed, lysed and laminin and protein contents in each sample was measured by the above mentioned method. Four sets of experiments were carried out.

Evaluation of the effect of control, Mf and morphine-Mf supernatants on synthesis of collagen

Equal numbers (10,000 cells/well) of mouse mesangial cells were plated in six-well plates. Cells were incubated in media containing RPMI, 5% FCS and 10% supernatants (control or Mf or morphine-Mf) for one week. Incubation medium was changed every other day. At the end of the incubation cells were washed, lysed and collagen type IV content was measured using the dot blot assay (in these assays we used anti collagen type IV antibodies in place of antilaminin antibodies). Four sets of experiments were performed.

Evaluation of the effect of anti-TGF-β antibodies on morphine-Mf supernatant-induced collagen synthesis

Equal numbers (6,000 cells/well) of mouse mesangial cells were plated in six well plates. Cells were incubated in media (RPMI + 5% FCS) containing either 10% Mf supernatant, 10% morphine-Mf supernatant or 10% morphine-Mf supernatant + anti-TGF-β antibodies (5 μg/ml) for one week. Incubation medium was changed every other day. At the end of the incubation period cells were washed, lysed and collagen contents were measured by the dot blot assay. Five series of experiments were carried out.

Mesangial RNA extraction and identification of TGF-β

Northern blot analysis of mRNA for TGF-β was performed on RPMI (control), 50% MSP and 50% M-MSP treated MC, grown in 100 mm cell culture dishes until confluent (one week). Total RNA was extracted from lysates of confluent MCs by the method of Chomczynski and Sacchi [20]. Aliquots of total RNA were treated with glyoxal and DMSO, electrophoresed in a 1% agarose gel, and transferred to nitrocellulose membranes. RNA ladder was included and the gel was stained with ethidium bromide to determine the size of RNA. cDNA probes specific for TGF-β (R & D Systems) was used for hybridization after [^{32}P]dCTP labeling by random-prime labeling. Filters were prehybridized at 50°C for three hours and then hybridized at 65°C for 16 hours with the labeled cDNA probe. The membranes were washed under stringent conditions with 2 × SSC and 1% SDS at 65°C. After washing, the membranes were kept in contact with XAR-5 film and intensifying screen at −70°C and developed. The membranes were washed to remove the hybridized probe and reprobed with GAPDH to ascertain that similar amounts of RNA were applied to the gel.

Effect of anti-TGF-β antibodies on mRNA expression for TGF-β on mesangial cells

To determine the effect of TGF-β on mRNA expression for TGF-β in mesangial cells we incubated confluent cells in incubation medium containing either buffer alone (control), 50% MSP, 50% MSP + anti-TGF-β antibody (5 μg/ml), 50% M-MSP, or 50% M-MSP + anti-TGF-β antibody (5 μg/ml) for 24 hours. At the end of the incubation period cells were washed and total RNA was extracted. An equal amount (20 μg) of RNA for each experimental condition was used to determine mRNA expression for TGF-β on mesangial cells by the above mentioned method.

Table 1. Cytotoxic effect of morphine on macrophages

	Control	Morphine		
		10^{-8} M	10^{-6} M	10^{-4} M
Absorbance	0.623 ± 0.010	0.655[b] ± 0.0.17	0.589 ± 0.032	0.531[a] ± 0.025

Macrophages were grown to confluence in a 96-well plate. Cells were washed twice with phosphate buffered saline and then reincubated in RPMI 1640 (no phenol red) with 10% FCS ± increasing concentrations of morphine (10^{-8} M to 10^{-4} M) for 24 hours. MTT in an amount equal to 10% of the final concentration of media was added to each well and the plate was incubated for a further two hours. At the end of the incubation period, formazan crystals were solubilized and the absorbency was measured at 540 nm to determine differences. Results represent means ± SEM from 16 wells for each variable. To compare values between multiple groups, analysis of variance was applied and a Newman-Keuls multiple range test was used to calculate a q value.

[a] $P < 0.05$ compared with control

[b] $P < 0.01$ compared with morphine 10^{-4} M

Statistical analysis

For comparison of mean values between two groups an unpaired *t*-test was used. To compare values between multiple groups analysis of variance (ANOVA) was applied. A Newman-Keuls multiple range test was used to calculate a q value. All values are reported as mean ± SEM except where otherwise indicated. Statistical significance was defined as $P < 0.05$.

Results

Fluorometric assay of morphine in supernatants

MSP as well as M-MSP did not show any presence of morphine. Thus, morphine does not seem to play a direct role in M-MSP-induced mesangial cell growth.

Cyotoxic effect of morphine on macrophages

The effect of morphine on macrophages is shown in Table 1. Morphine in concentrations of 10^{-6} to 10^{-8} M did not show any cytotoxic effect on macrophages. However, morphine in a higher concentration (10^{-4} M) showed decreased mitochondrial dehydrogenases activity (Table 1).

Effect of interaction between morphine and macrophages on mesangial cell proliferation

Macrophage secretory products (MSP) significantly enhanced ($P < 0.001$) RMC proliferation when compared with control cells (control, 127.3 ± 3.4 vs. 453.1 cpm/μg protein). Mesangial cells that were incubated with M-MSP demonstrated significantly higher levels ($P < 0.01$) of [^{3}H]thymidine incorporation when compared with RMC treated with MSP (MSP, 453.1 ± 25.6 vs. M-MSP, 591.6 ± 30.1 cpm/μg protein). These results indicate that macrophage secretory products enhance RMC proliferation. Activation of macrophages by morphine appears to further enhance the mitogenic effect of MSP on MC proliferation.

In another set of experiments, RMCs after growth arrest were incubated in RPMI 1640 alone (CSP), RPMI 1640 containing 1% fetal calf serum (FCS), RPMI 1640 + 1% FCS + MSP (FCS-MSP) or RPMI 1640 + 1% FCS + M-MSP (FCS-MMSP) for 24 hours and pulsed with [^{3}H]thymidine. The effect of FCS, FCS-MSP and FCS-M-MSP on RMC proliferation is shown in Figure 1. FCS increased (sixfold) DNA synthesis when compared to control cells. Morphine treatment of macrophages (FCS-MMSP) enhanced ($P < 0.01$) RMC proliferation when compared with FCS or FCS-MSP (FCS-MSP, 834.6 ± 32.0 vs. FCS-MMSP, 1,170 ± 50.3 cpm/μg protein). These results suggest that M-MSP has an additive action on the growth promoting effect of fetal calf serum.

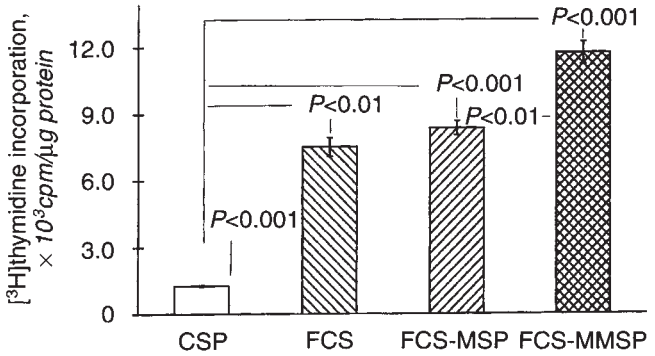

Fig. 1. *Comparison of the effect of FCS, MSP and M-MSP on mesangial cell proliferation.* Mesangial cells that were grown to subconfluence and growth arrested for 72 hours were incubated either with RPMI 1640 alone (CSP), RPMI 1640 + 1% FCS (FCS), RPMI 1640 + 1% FCS + 50% MSP (FCS-MSP), or RPMI 1640 + 1% FCS + 50% M-MSP (FCS-MMSP) for 48 hours. A 0.25 μCi aliquot of [^{3}H]thymidine was added to each well and thymidine incorporation as a marker of DNA synthesis was determined. Results represent mean ± SEM from six series of experiments carried out in triplicate. A Newman-Keuls multiple range test was used to calculate a q value.

Table 2. Effect of morphine treated mouse macrophage supernatant on mouse mesangial cell proliferation

	Mouse macrophage supernatants					
	5%		10%		20%	
	MϕSP	M-MϕSP	MϕSP	M-MϕSP	MϕSP	Mϕ-MSP
Cell count 10^4 cells/well	22.5 ± 0.6	22.4 ± 0.7	22.9 ± 0.3	36.3[a] ± 2.0	23.1 ± 0.2	41.8[b,c] ± 0.4

Equal numbers of mouse mesangial cells were grown to semiconfluence on 24-well plates and then growth arrested for 72 hours. Subsequently, cells were washed twice with PBS and incubated in RPMI containing variable concentrations (5%, 10% and 20%) of either MϕSP or M-MϕSP for 48 hours at 37°C. At the end of the incubation period cells were washed, trypsinized and counted in a hemocytometer. Results represent means ± SEM from three sets of experiments performed in quadruplicate. A Newman-Keuls multiple range test was used to calculate a q-value.

[a] $P < 0.01$ compared with 10% MϕSP and 5% M-MϕSP

[b] $P < 0.001$ compared 20% MϕSP and 5% M-MϕSP

[c] $P < 0.05$ compared 10% M-MϕSP

Effect of M-MϕSP on proliferation of mouse mesangial cells

The effect of M-MϕSP on mouse mesangial cell proliferation is shown in Table 2. M-MϕSP enhanced proliferation of mouse mesangial cells at 10% and 20% concentrations. This effect of M-MϕSP was dose dependent. Since the mitogenic effect of M-MϕSP was obvious at lower concentrations (10% concentration), it appears that perhaps M-MϕSP has a higher concentration of growth promoting factors.

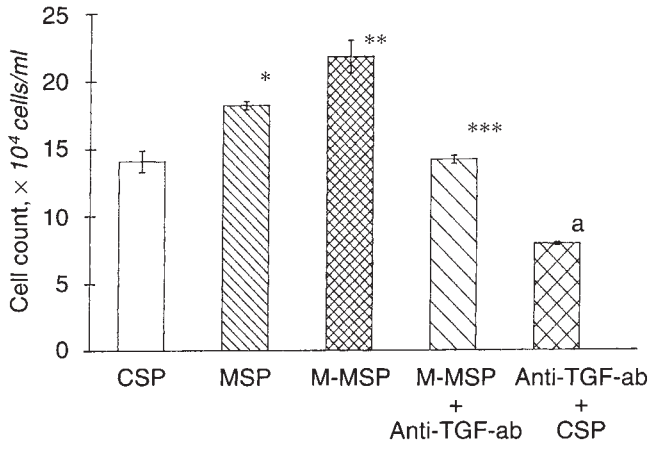

Fig. 2. *Effect of anti-TGF-β antibody on mesangial cell proliferation.* Subconfluent MC were growth arrested for 72 hours. Cells were incubated with RPMI containing either buffer alone (control, CSP), 50% MSP, 50% M-MSP, 50% M-MSP + anti-TGF-β antibody (5 μg/ml), or anti-TGF-β antibody (5 μg/ml) for 48 hours. At the end of the incubation period, cells were washed, trypsinized and counted in a hemocytometer. Results (means ± SEM) are from three series (each carried out in triplicate) of experiments. To compare values between multiple groups analysis of variance (ANOVA) was applied. A Newman-Keuls multiple range test was used to calculate a q value. $^{*}P < 0.01$ compared with control; $^{**}P < 0.001$ compared with control; $^{a}P < 0.001$ compared with CSP; $^{***}P < 0.001$ compared with M-MSP.

Table 3. Effect of anti-TGF-β antibody on M-MSP-induced mesangial cell proliferation

	Control	MSP	M-MSP	M-MSP+ anti-TGF-β ab	Anti-TGF-β ab
Cell count	14.1	18.2[a]	21.8[bc]	14.2[cd]	7.9[b]
× 10^4 cells/ml	±	±	±	±	±
	0.8	0.3	1.2	0.3	0.1

Equal number of mouse mesangial cells were plated in 24-well plates. After 3 to 4 days, subconfluent MC were growth arrested for 72 hours. Subsequently cells were incubated with RPMI containing either buffer alone (control), 50% MSP, 50% M-MSP, 50% M-MSP + anti-TGF-β antibody (5 μg/ml), or anti-TGF-β antibody (5 μg/ml) for 48 hours. At the end of the incubation period, cells were washed, trypsinized and counted in a hemocytometer. Results (means ± SEM) are from three sets of experiments, each carried out in triplicate. To compare values between multiple groups analysis of variance (ANOVA) was applied. A Newman-Keuls multiple range test was used to calculate a q value.

[a] $P < 0.01$ compared with control
[b] $P < 0.001$ compared with control
[c] $P < 0.05$ compared with MSP
[d] $P < 0.001$ compared with M-MSP

Effect of anti-TGF-β antibody on M-MSP-induced mesangial cell proliferation

The effect of anti-TGF-β antibody on M-MSP-induced MC proliferation is shown in Figure 2 and Table 3. M-MSP enhanced ($P < 0.001$) mesangial cell proliferation when compared with control (control, 14.1 ± 0.8 vs. M-MSP, 21.8 ± 1.2 × 10^4 cells/ml). Anti-TGF-β antibody attenuated the effect M-MSP on mesangial cell growth (Table 3).

Effect of naloxone on M-MSP-induced mesangial cell proliferation

The effect of naloxone on M-MSP-induced mesangial cell proliferation is shown in Table 4. Naloxone did not attenuate the mitogenic effect of M-MSP on mesangial cells (control, 9.4 ± 1.0, M-MSP, 16.0 ± 0.2, naloxone + M-MSP, 13.6 ± 1.6 × 10^4 cells/ml).

Table 4. Effect of naloxone on M-MSP induced mesangial cell proliferation

	MSP	M-MSP	N + M-MSP
Cell count	9.4	16.0[a]	13.6
× 10^4 cells/ml	±	±	±
	1.0	0.2	1.6

Equal numbers of mouse mesangial cell were plated in 24-well plates and grown to subconfluence. MMC were growth arrested for 72 hours. Cells were washed twice with PBS and incubated in RPMI containing either 20% MSP, 20% M-MSP, or 20% N-M-MSP for 48 hours. At the end of the incubation period, cells were trypsinized and counted in a hemocytometer. Results (means ± SEM) are from three series of experiments, each performed in triplicate. To compare values between multiple groups analysis of variance (ANOVA) was applied. A Newman-Keuls multiple range test was used to calculate a q value.

[a] $P < 0.05$ compared with MSP

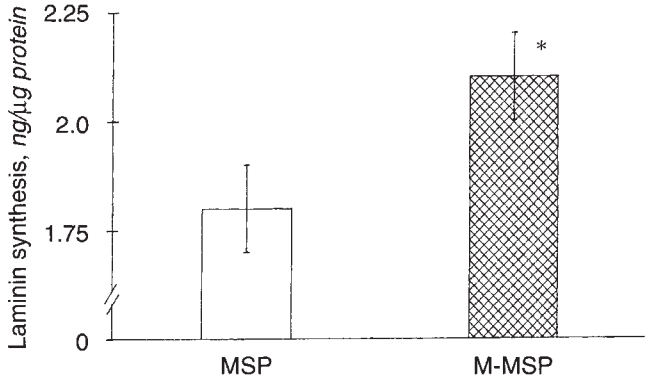

Fig. 3. *Effects of M-MSP on the synthesis of laminin.* Equal numbers of mesangial cells were plated and incubated in growth medium containing either 50% MSP (MSP) or 50% M-MSP (M-MSP) for two weeks. Media was changed twice a week. At the end of two weeks cells were harvested and the amount of laminin was determined by a dot blot assay. Results represent means ± SEM (ng/μg cell protein) from four series of experiments carried out in triplicate. $^{*}P < 0.05$ compared to MSP.

Effect of MSP and M-MSP on synthesis of laminin

The effect of MSP and M-MSP on laminin synthesis by RMC is shown in Figure 3. M-MSP increased ($P < 0.05$) mesangial synthesis of laminin when compared with cells treated with MSP alone. These results indicate that morphine increases laminin synthesis by RMC.

Dose response effect of MSP and M-MSP

The comparable effect of variable concentrations (10% to 70%) of MSP and M-MSP on the synthesis of laminin is shown in Table 5. M-MSP enhanced the synthesis of laminin at concentrations of 10% to 50% when compared to MSP alone. A dose response effect of M-MSP on laminin synthesis by RMC is shown in Figure 4. M-MSP increased synthesis of laminin in a dose dependent manner. M-MSP increased maximal synthesis of laminin by RMC at a concentration of 50%.

Table 5. Dose response effect of morphine and macrophage interaction on laminin synthesis by mesangial cells

% Supernatant variable	10%		30%		50%		70%	
	MSP	M-MSP	MSP	M-MSP	MSP	M-MSP	MSP	M-MSP
Mean *ng/mg protein*	99.0	174.0[a]	127.0	195.0[b]	126.0	312.0[a]	168.0	235.0
SEM	6.0	5.0	16.0	13.0	19.0	14.0	39.0	30.0

Equal numbers of mesangial cells were plated on six-well plates in incubation medium containing RPMI 1640 + 2.5% fetal calf serum and variable concentrations (10% to 70%) of control or morphine treated macrophage supernatant and cells were allowed to grow for two weeks. Media was changed every third day. At the end of the incubation cells were scraped from each well and a dot blot assay carried out to measure the total amount of laminin. Results represent means ± SEM from four sets of experiments.

[a] $P < 0.001$ compared with respective controls

[b] $P < 0.02$ compared with respective control

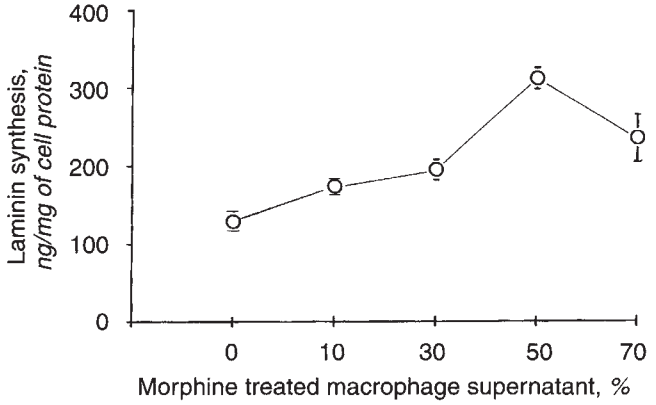

Fig. 4. *Dose response effect of M-MSP on the synthesis of laminin.* Equal numbers of mesangial cells were plated on six-well plates. Cells were incubated in growth medium containing 0 to 70% morphine treated macrophage supernatant for two weeks. At the end of the incubation period, cells were harvested and the amount of laminin was measured by dot blot assay. Results represent means ± SEM (ng/mg of protein) from four series of experiments.

Table 6. Effect of morphine-Mϕ supernatant on synthesis of laminin by mouse mesangial cells

	Control supernatant	Mϕ supernatant	Morphine-Mϕ supernatant
Laminin *ng/mg protein*	919.1 ± 20.2	1379.4 ± 68.3[a]	1999.8 ± 79.4[b]

Equal number of mouse mesangial cells were grown in medium containing either 20% control supernatant, 20% Mϕ supernatant, or 20% morphine-Mϕ supernatant for one week. The medium was changed every 3rd day. At the end of the incubation period cells were washed, lysed and measurement of laminin and protein was carried out. Results (means ± SEM) are from 4 sets of experiments. To compare values between the groups analysis of variance (ANOVA) was applied. A Newman-Keuls multiple range test was used to calculate a q value.

[a] $P < 0.01$ compared with control

[b] $P < 0.001$ compared with control and Mϕ supernatant

Table 7. Effect of morphine-Mϕ supernatant on synthesis of collagen type IV by mouse mesangial cells

	Control supernatant	Mϕ supernatant	Morphine-Mϕ supernatant
Collagen type IV *ng/mg protein*	1698.7 ± 81.6	1735.7 ± 93.1	2412.9 ± 121.2[a]

Equal number of mouse mesangial cells were grown in medium containing either 10% control supernatant, 10% Mϕ supernatant, or 10% morphine-Mϕ supernatant for one week. Incubation medium was changed every other day. At the end of the incubation period cells were washed, lysed and collagen type IV contents were measured (dot blot assay). Results represent means ± SEM from 4 series of experiments. To compare values between the groups analysis of variance (ANOVA) was applied. A Newman-Keuls multiple range test was used to calculate a q value.

[a] $P < 0.01$ compared with control and Mϕ supernatants

Effect of M-MϕSP on laminin synthesis by mouse mesangial cells

The effect of M-MϕSP on the synthesis of laminin by mouse mesangial cells (MMC) is shown in Table 6. MϕSP enhanced ($P < 0.01$) the synthesis of laminin by MMC. M-MϕSP increased ($P < 0.001$) synthesis of laminin when compared with control cells as well as MSP treated cells.

Effect of M-MϕSP on collagen synthesis by mouse mesangial cells

The effects of control, MϕSP and M-MϕSP on the synthesis of collagen type IV by mouse mesangial cells are shown in Table 7.

Mouse mesangial cells treated with M-MϕSP showed a greater ($P < 0.01$) amount of collagen synthesis when compared with control and MϕSP treated cells.

Effect of anti-TGF-β antibodies on M-MϕSP-induced collagen synthesis

The effect of anti-TGF-β antibodies on M-MϕSP-induced collagen synthesis is shown in Figure 5. M-MϕSP enhanced ($P < 0.05$) the synthesis of collagen by MMC when compared to MϕSP treated cells. However this effect of M-MϕSP was inhibited ($P < 0.05$) when cells were also treated with anti-TGF-β antibodies (MSP, 1149.4 ± 35.7, M-MϕSP, 1515.1 ± 82.7, anti-TGF-β antibodies + M-MϕSP, 1068.9 ± 131.0 ng collagen/mg protein). These results indicate that M-MϕSP-induced enhanced synthesis of collagen may be mediated through TGF-β.

Effect of MSP and M-MSP on mRNA expression for TGF-β on mesangial cells

The effect of control supernatant (CSP), MSP and M-MSP on mRNA expression for TGF-β on RMC is shown in Figure 6. RMC treated with M-MSP showed increased expression of mRNA for TGF-β when compared with mesangial cells treated with MSP or CSP. These results suggest that morphine activates macrophages to induce enhanced synthesis of TGF-β by mesangial cells.

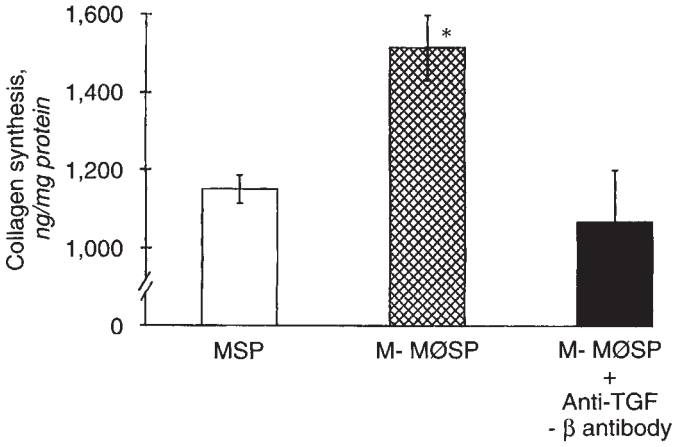

Fig. 5. *Effect of anti-TGF-β antibodies on M-MϕSP-induced collagen synthesis.* Equal numbers of mouse mesangial cells were incubated in medium (RPMI + 5% FCS) containing either 10% MSP, 10% M-MϕSP, or anti-TGF-β antibodies (5 μg/ml) + 10% M-MϕSP for one week. At the end of the incubation period cells were lysed and collagen contents were measured. Results represent means ± SEM from 5 sets of experiments. To compare values between multiple groups, analysis of variance was applied and A Newman-Keuls multiple range test was used to calculate a q value. $^*P < 0.05$ compared with MSP and M-MϕSP + anti-TGF-β antibodies.

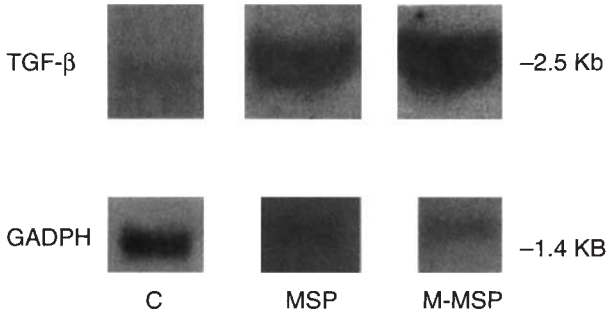

Fig. 6. *Northern blots of TGF-β (upper panel) and GAPDH (lower panel).* mRNA expression on mesangial cells after a one week exposure to incubation medium alone (C), incubation media containing either 50% macrophage supernatant (MSP) or 50% morphine treated macrophage supernatant (M-MSP).

Effect of anti-TGF-β antibodies on mRNA expression for TGF-β on mesangial cells

The effect of anti-TGF-β antibodies on MSP and M-MSP induced mRNA expression for TGF-β on MC is shown in Figure 7. Densitometric evaluation of these bands did not show any decrease in mRNA expression for TGF-β on MC in anti-TGF-β antibody treated samples.

Discussion

The present study clearly demonstrates that morphine-induced macrophage secretory products stimulates proliferation of rat as well as mouse mesangial cells. Morphine-induced macrophage products also enhanced the synthesis of laminin by both types of mesangial cells. This effect of M-MSP was dose dependent. M-MϕSP also enhanced the synthesis of type IV collagen by mouse mesangial cells. This effect of M-MϕSP on collagen

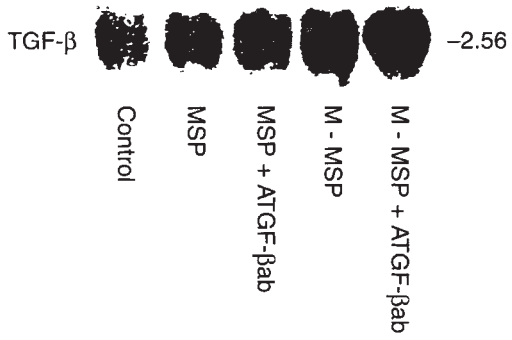

Fig. 7. *Northern blots for mRNA expression on mesangial cells TGF-β (2.56 kb).* Confluent mouse mesangial cells were incubated either with buffer (control), 50% MSP, 50% MSP + anti-TGF-β antibody (ATGF-βab, 5 μg/ml), 50% M-MSP, or anti-TGF-β antibody (5 μg/ml) + 50% M-MSP for 24 hours. Total RNA was extracted and 20 μg of RNA was used for each lane.

synthesis was attenuated by anti-TGF-β antibodies. Moreover, the interaction between morphine and macrophages also enhanced the expression of mRNA for TGF-β on mesangial cells. It appears that the effect of M-MSP on MC may be mediated through the generation of TGF-β by MC.

There is increasing evidence which suggest that macrophage infiltration contributes to the progression of glomerulosclerosis [4–9]. Saito and Atkins in the experimental model of puromycin aminonucleoside (PAN)-induced nephrosis showed significant infiltration of macrophages in the glomeruli when the sclerotic lesion appeared [21]. In the PAN model, depletion of glomerular macrophages by essential fatty acid deficient diets ameliorated acute renal dysfunction [7]. In the same model, timed systemic X-irradiation also attenuated the late development of glomerulosclerosis [4, 5]. These authors emphasized the role of macrophages in the development of FGS in the PAN model. Van Goor et al [8] demonstrated glomerular macrophage influx as a major structural alteration associated with FGS in the setting of renal ablation. In their later experiments these investigators induced sustained glomerular macrophage depletion in remnant kidney rats by a regimen of sublethal triple irradiation. In these experiments, histologic examination showed a significant decrease in mesangial cell proliferation and mesangial matrix expansion in glomeruli of X-irradiated rats when compared with non-irradiated remnant kidney rats [9]. However, in the late stage in X-irradiated rats glomeruli showed influx of macrophages which was associated with the expansion of the mesangium [9]. Thus macrophages seem to play an important role in the progression of FGS in models of PAN-induced nephrosis and renal ablation.

Cytokines have been considered to be important mediators in macrophage associated glomerular injury [6, 22]. TGF-β has been shown to play a significant role in the pathogenesis of anti-Thy 1.1-induced mesangial expansion [22]. Ding, Pesek-Diamond [6] in the model of PAN-induced nephrosis showed that increased glomerular macrophage influx was also associated with increased glomerular mRNA expression for TGF-β1. Other macrophage cytokines which have evidence of contributing to glomerular inflammation are TNF-α and IL-1. However, these cytokines (TNF and IL-1) have been demonstrated to play an important role in proliferative glomerulonephritis [22]. Their role in FGS is not clear.

TGF-β has been isolated from bovine kidney [23], rat kidney fibroblasts [24] and mesangial cells [25]. Glomerular cells have been demonstrated to express specific receptors for TGF-β [25]. Although TGF-β inhibited proliferation of glomerular endothelial and epithelial cells it acted as a bifunctional regulator of mesangial cell proliferation [25]. TGF-β has also been shown to have a direct effect on the synthesis of mesangial matrix [22]. Since other growth factors produced by macrophages including, interleukin-1 and tumor necrosis factor, have been demonstrated not to have a significant effect on the synthesis of matrix components [22], we believe that the M-MSP induced effect on the synthesis of matrix components was related to the increase in the production of TGF-β by activated macrophages.

The effects of morphine on immunomodulation have been shown to be mediated through opiate receptors on monocytes and lymphocytes [26, 27]. The molecular mechanism responsible for the effect of morphine is not clear. However, TGF-β antibodies inhibited the effect of opiates on the respiratory burst activity in porcine peripheral blood monocyte cultures [28]. TGF-β has also been reported to be a potent monocyte/macrophage inhibitor of respiratory burst activity of these cells in many species, including rodents, and swine [27, 29]. All these studies suggest that TGF-β may be a mediator of some of the effects exerted by opiates on monocytes/macrophages. Recently, Chao et al [30] demonstrated that morphine enhances the release of TGF-β by mononuclear cell cultures. The present study showing enhanced mRNA expression for TGF-β on mesangial cells by morphine treated macrophage secretory products provides a basis for speculation by which morphine can contribute to the expansion of the mesangium.

The development of glomerulosclerosis in patients with heroin addiction has been attributed to a variety of factors [31–36]. The majority of patients with heroin nephropathy are black [31]. It has been suggested that the Negroid race has a genetic predisposition to develop heroin nephropathy [32]. Recently, Friedman and Rao reported a decline in the prevalence of heroin nephropathy in their hospital [33]. These authors attributed this decline to the availability of pure heroin in the market. These investigators suggested that heroin impurities may be playing a role in the higher incidence of heroin nephropathy in the past. However, a significant number of patients with HIV infection who develop focal glomerulosclerosis are also drug addicts [34]. It appears that drugs and HIV infection may be playing a synergistic role in the development of glomerulosclerosis. Both drugs and HIV modulate the immune system and predispose persons to hepatitis and opportunistic infections [27, 35]. Many of these infectious agents including hepatitis B and cytomegalo virus have been demonstrated to cause damage to the kidney [36].

We conclude that morphine-induced macrophage secretory products stimulate proliferation of mesangial cells. They also enhance the synthesis of laminin and collagen type IV by mesangial cells. This action of morphine on mesangial cells seems to be partly mediated through TGF-β. The present study suggests a possible role for macrophages in the development of glomerular lesions in patients with drug addiction.

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